IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	ATTY.'S DOCKET: BARENHOLZ=1
Yechezkel BARENHOLZ et al.	Art Unit: 1637
	Examiner: FREDMAN, Jeffrey Norman
Appln. No.: 09/780,757	
Filed: August 2, 2001	Washington, D.C.
For: DETECTION OF BINDING OF CHARCED SPECIES USING PH OR POTENTIAL SENSITIVE PROBES	Confirmation No. 6619

DECLARATION UNDER 37 CFR 1.132

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Sir:

I, Yechezkel BARENHOLZ, do hereby state and declare as follows:

I am an inventor of the above-identified application and my educational and professional experience is provided in the CV attached hereto as Exhibit A.

The present invention describes methods for detecting binding of species to a given surface having a defined pH or surface potential. Specifically, a probe which comprises a pH and/or potential sensitive fluorophore is stably incorporated at the surface, and a change in a fluorescence is observed upon binding (or dissociation) of the

species at the surface due to a change in surface potential or pH. The change in fluorescence thus serves as an indicator for the association and dissociation between the surface and the species.

As stated in page 2 of the specification, the surface may be a lipid bilayer (line 12) or a polymer (line 30). In case the surface is a polymer, the fluorophore is stably bound to the surface by covalent linkage (line 31). Such polymer may be in various forms such as micro- or nanoparticles, or as sheets; e.g., cellulose-based polymers (lines 33-34). The sentence bridging pages 8 and 9 of the specification list many other non-lipid polymers that may be used as substrates for the present invention, including polysaccharide polymers, such as dextrans and cellulose-based polymers, polyacrylates and polymethacylrates, polyesters, polyethers, polyamines, polyamides, polyimides, polystyrenes, polyamino acids, such as polylysine or polyarginine, and fluoropolymers. Such polymers may be in various forms such as micro- or nanoparticles, fibers, or sheets.

Thus, the specification makes clear that the method is applicable for various polymers. Furthermore, the specification provides working examples with dextran as a surface to which fluorescein was covalently attached to form a dextran-fluorescein conjugate, as well a with an oligonucleotide-fluorescein conjugate. Figs. 12A-12B relating to these specific examples show the pH dependent fluorescence of these conjugates.

Fig. 13 illustrates the change in fluorescence of FITC-labeled oligonucleotide (Bcl2-TIAS) on addition of increasing amounts of liposomes composed of DOTAP:DOPE, DOTAP

and DOPE/DOPC, respectively. As shown, the change in fluorescence of the conjugate comprised of a negatively charged oligonucleotide was indicative of the binding of the positively charged liposomes.

Attached hereto as Exhibit B is a print-out of a Power-Point presentation showing results with two cellulose based polymers, dextran-spermine conjugate and arabinogalactan-spermine conjugate, both covalently attached to hydroxycoumarin.

Slide **1** is a general structure of a polysaccharide-spermine (polycation) as used in the examples described in this exhibit.

Slide **2** is a chemical scheme for the synthesis procedure for obtaining polysaccharide-spermine.

Slide **3** provides the chemical composition of polysaccharide-spermine conjugates.

Slide **4** provides the chemical characterization of the polysaccharide-spermine conjugates.

Slide **5** provides the electrostatics of polysaccharide-spermine conjugates determined through covalently attached hydroxycoumarin.

Slide **6** provides the electrostatics of polysaccharide-spermine conjugates.

Slide **7** shows electrostatics neutralization by DNA of polysaccharide-spermine conjugates covalently attached to hydroxycoumarin.

Slide 8 shows that the electrostatics neutralization by DNA of polysaccharide-spermine conjugates covalently attached to hydroxycoumarin is dose dependent.

It is thus evident from Slides 7 and 8 that the potential of the surface of the polysaccharide-spermine conjugates is altered when DNA associates with the surface.

Similar experiments have now been conducted with a protein-based surface, the results of which are presented in Exhibit C. Specifically, albumin microparticles were labeled with FITC to form FITC-albumin surface. The FITC-albumin was used as pH surface potential probe. A significant change (≤30%) in fluorescein fluorescence intensity occurred when cationic liposomes (DOTAP) or cationic polymers polyethyleneimine (PEI) or polylysine (i.e. species to be detected), were used. No change occurred when neutral or anionic liposomes were used.

The experiments reported in Exhibits B and C and discussed herein were all conducted under my supervision and thus I have first-hand knowledge of the results thereof.

Thus, the examples provided in the specification and in the attached exhibits provide evidence that the method of the invention is applicable for a variety of polymers.

Procedures for fluorescence labeling of polymers were well available at the time of the invention. For example, Hudson, L. and Hay, F.C., *Practical Immunology*, Blackwell Scientific Publications, Oxford, 1989, describe procedures for labeling antibodies. In addition, Brinkley, M., "A brief survey of methods for preparing protein conjugates with dyes, heptans and cross-linking reagents" *Bioconjug. Chem.*, 3:2-13 (1992), provides techniques for

labeling proteins. Yet, in addition, John J. Hill et al. Fluorescence Approaches to Study Protein-Nucleic Acid Complexation, *Methods Enzymology* 278:390-416, (1997) describe labeling of oligonucleotides and proteins. Variations of these and other techniques were also available at the time of the invention, e.g., as described by Diminsky, D. et al. *Vaccine*, 15:637-647 (1997).

Numerous fluorophores were also available at the time the application was filed. Exhibit D, attached hereto, includes the table of content and some exemplary pages from a handbook of Fluorescent and probes issued on 1996, showing the availability of the probes and their applicability as biopolymer markers [HandbooK of Fluorescent Probes and Research Chemicals, Richard P. Haugland, 6th edition, MOLECULAR PROBES, 1996]

The examples provided in the application, in combination with the art available at the time, should have provided fair basis for the successful performance of the method of the invention with other polymers. The underlying concept of the method of the invention is that as long as a probe is stably incorporated (e.g., by anchoring, covalent linkage etc.) into or onto a surface (the surface having local environment at a given pH or surface potential) any change in the pH or potential of the surface, as a result of association or dissociation of a species to the surface, may be detected by the change in the observable property the probe. This conclusion is further supported by the examples attached to this declaration.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements

made on information and belief are believed to be true; and further that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 81 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon:

Yechezkel BARENHOLZ

June 22. 2005
Date

BARENHOLZ'S CV

EXHIBIT A

November 2004

Curriculum Vitae

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27 April 1941: Born in Tel-Aviv, Israel
Married, 4 daughters

1955-1959: Graduate High School
1959-1962: Military Service (Israeli army)

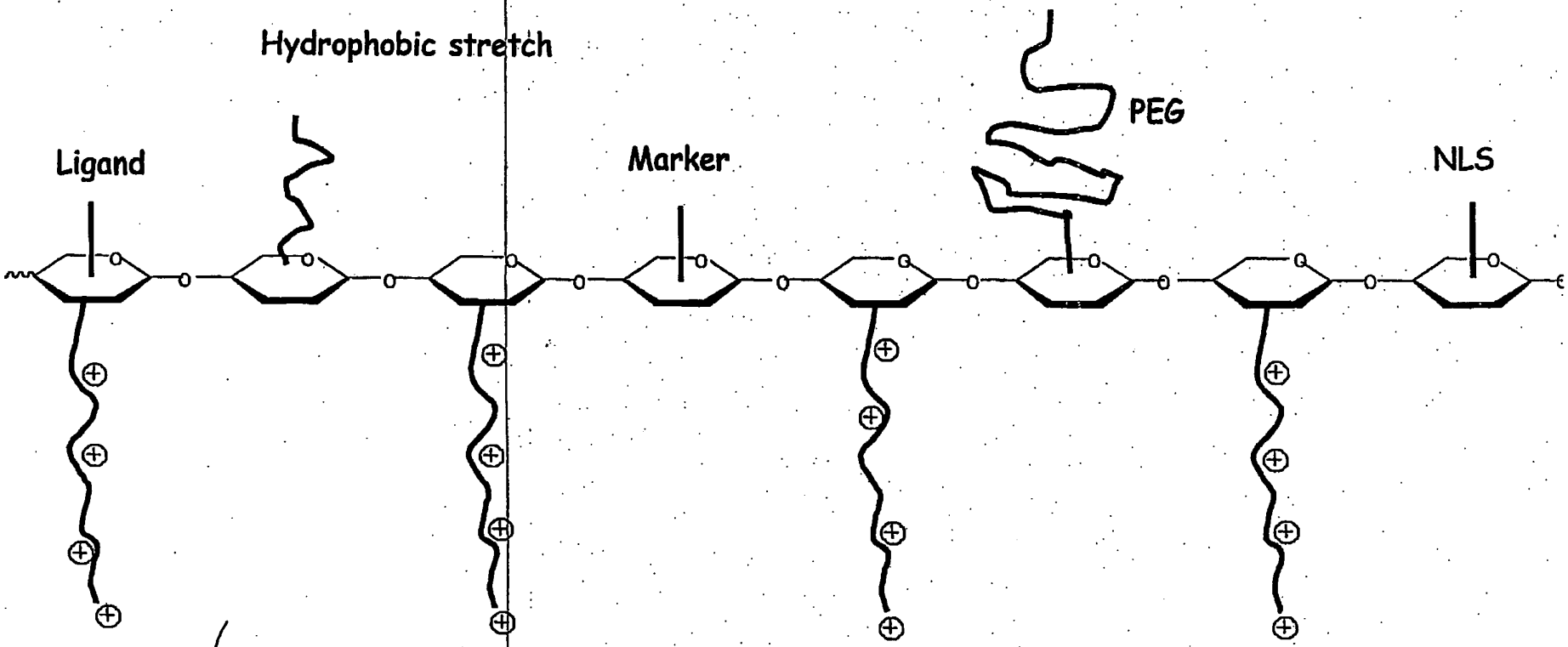
1965 : B.Sc. in Biochemistry and Microbiology, The Hebrew University, Jerusalem, Israel.
1965 : Research Assistant, Department of Biochemistry, The Hebrew University – Hadassah Medical School, Jerusalem, Israel.
1968 : M.Sc. in Biochemistry (*cum laude*), The Hebrew University of Jerusalem, Israel.
1967-1969 : Assistant, Department of Biochemistry, The Hebrew University – Hadassah Medical School, Jerusalem, Israel.
1969 : Research with Drs. R.M.C. Dawson and A. Bangham, A.R.C., Institute of Animal Physiology, Babraham, Cambridge, England, British Council fellowship.
1969-1971 : Instructor, Department of Biochemistry, The Hebrew University – Hadassah Medical School, Jerusalem, Israel.
1968-1971 : Ph.D. Candidate, Department of Biochemistry, Sponsor: Professor S. Gatt, The Hebrew University–Hadassah Medical School, Jerusalem, Israel.
1971 : Ph.D., Biochemistry, The Hebrew University, Jerusalem, Israel.
1971-1973 : Lecturer in Biochemistry, Department of Biochemistry, The Hebrew University – Hadassah Medical School, Jerusalem, Israel.
1973-1982 : Visiting Assistant and Associate Professor of Biochemistry, Department of Biochemistry, University of Virginia Medical School, Virginia, U.S.A.
1974-1978 : Senior Lecturer in Biochemistry, Department of Biochemistry, The Hebrew University – Hadassah Medical School, Jerusalem, Israel.
1978-1982 : Associate Professor of Biochemistry, Department of Biochemistry, The Hebrew University – Hadassah Medical School, Jerusalem, Israel.
1983-1997: Visiting Professor of Biochemistry, University of Virginia Medical School, Charlottesville, VA, U.S.A.
1982-present: Professor of Biochemistry, Department of Biochemistry, The Hebrew University – Hadassah Medical School, Jerusalem, Israel.

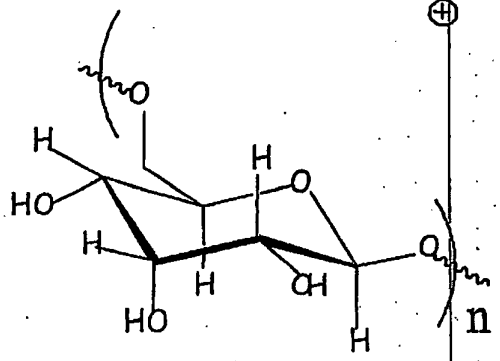
1991 : Honored as **Donders Chair Professor,** Utrecht University, The Netherlands.
Special award for excellent contributions to the field of liposome science.
1995 : FDA Approval of DOXIL (DOX-SL) (Publication Nos: 176, 179, 184, 186, 191, 195, 196) and U.S. Patent No. 4.898.735, and 5.192.549, 5.316.771.
1995 : Kaye Award for Innovation (Hebrew University).
Subject: A novel approach to obtain efficient and stable remote drug loading of liposomes for clinical use.
1997 : Kaye Award for Innovation (Hebrew University).
Subject: Development of liposomal doxorubicin (DOXIL) for cancer treatment: from basic research to FDA approval.
1998 Visiting Professor (*invited*), University of Kyoto, Japan.
1998 : **Alec D. Bangham Achievement Award for life-long achievement resulting in fundamental and sustained impact on the advancement of liposome science and technology**
2000 : **TEVA Founder Prize for major contributions to Medical Biotechnology**
2003 Establishing **The Barenholz Prizes For Applied Research** to Ph.D. students in Israel for Excellency and innovation (from DOXIL royalties).

Executive Editor: Progress in Lipid Research
Editorial Board Member: Chemistry and Physics of Lipids
Journal of Liposome Research
Cellular & Molecular Biology Letters
International Journal of Oncology

(3)

Our modular biodegradable polycation

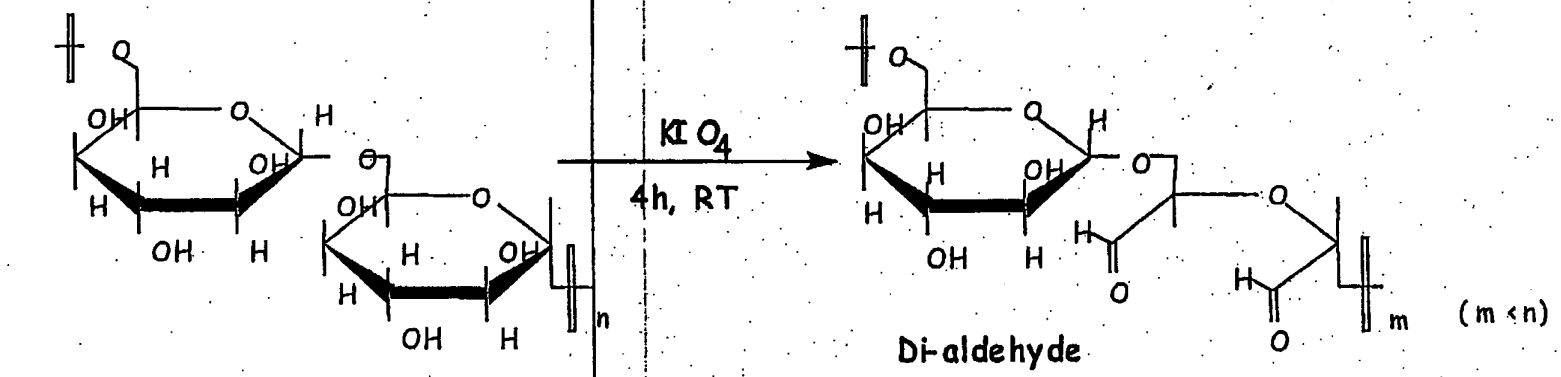


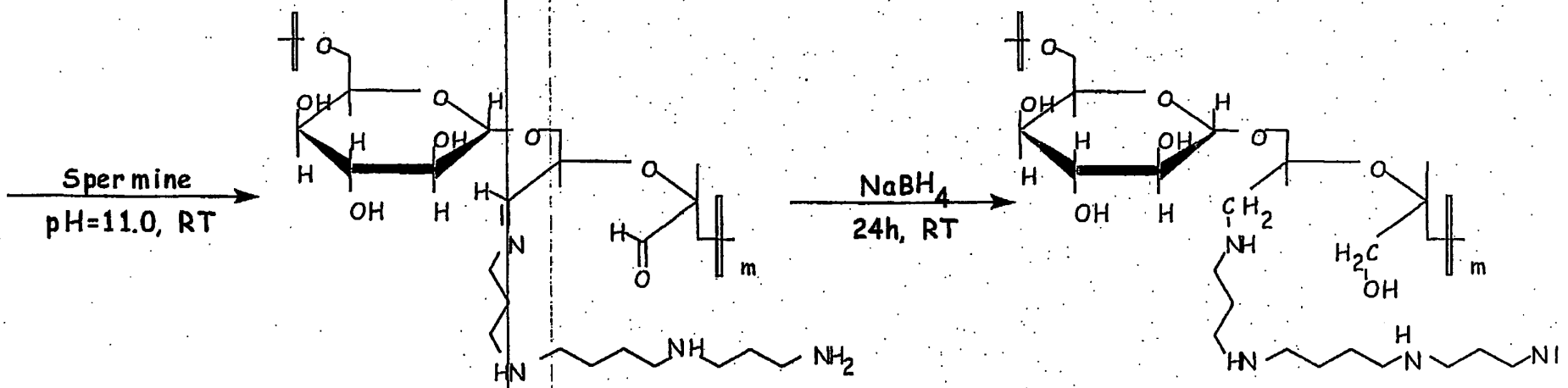


Dextran

- Biodegradable backbone
- Control of cationic density & distribution
- Multifunctional

Novel cationic polysaccaride system





(B)

Chemical composition of polysaccharide-spermine conjugates

Polymer code	chemical composition (Saccharide/KIO_4)
TA1-126A	Arabinogalactan(1:1)-Spermine
TA1-127A	Arabinogalactan(1:3)- Spermine
TA1-127B	Arabinogalactan(1:5)- Spermine
TA1-129A	**Dextran(1:1)- Spermine**
TA1-129B	Dextran(1:3)- Spermine

	DOTAP/Cholesterol (1:1)

(B)

Chemical characterization of conjugates

Polymer code	Composition	Nitrogen[a] (% weight)	Primary amino groups[b] (nmole/mg)	Spermine moieties[c] (nmole/mg)	Secondary amino groups[d] (nmole/mg)	Crosslinkec spermine[e] (%)
TA1-126A	AG(1:1)-S	8.31	1600	1480	4400	0
TA1-127A	AG(1:3)-S	3.91	750	700	2100	0
TA1-127B	AG(1:5)-S	2.66	520	480	1440	0
TA1-129A	**DEX(1:1)-S**	**11.19**	**1100**	**2000**	**6000**	**45**
TA1-129B	DEX(1:3)-S	4.85	890	870	2610	0

(a) determined by elemental analysis

(b) determined by TNBS method

(c) calculated from elemental analysis (total Nitrogen divided by 4)

(d) calculated from spermine (spermine content multiply by 3)

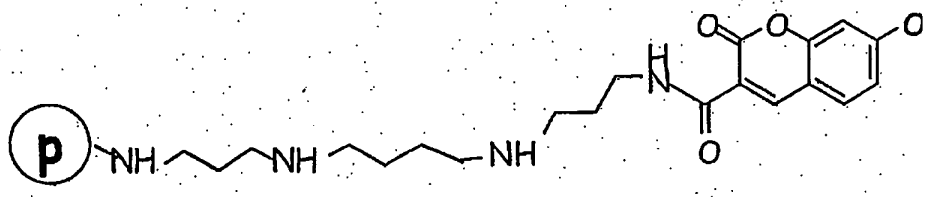
(e) (Total Nitrogen - primary amino groups - secondary amino groups)*100/(sperm

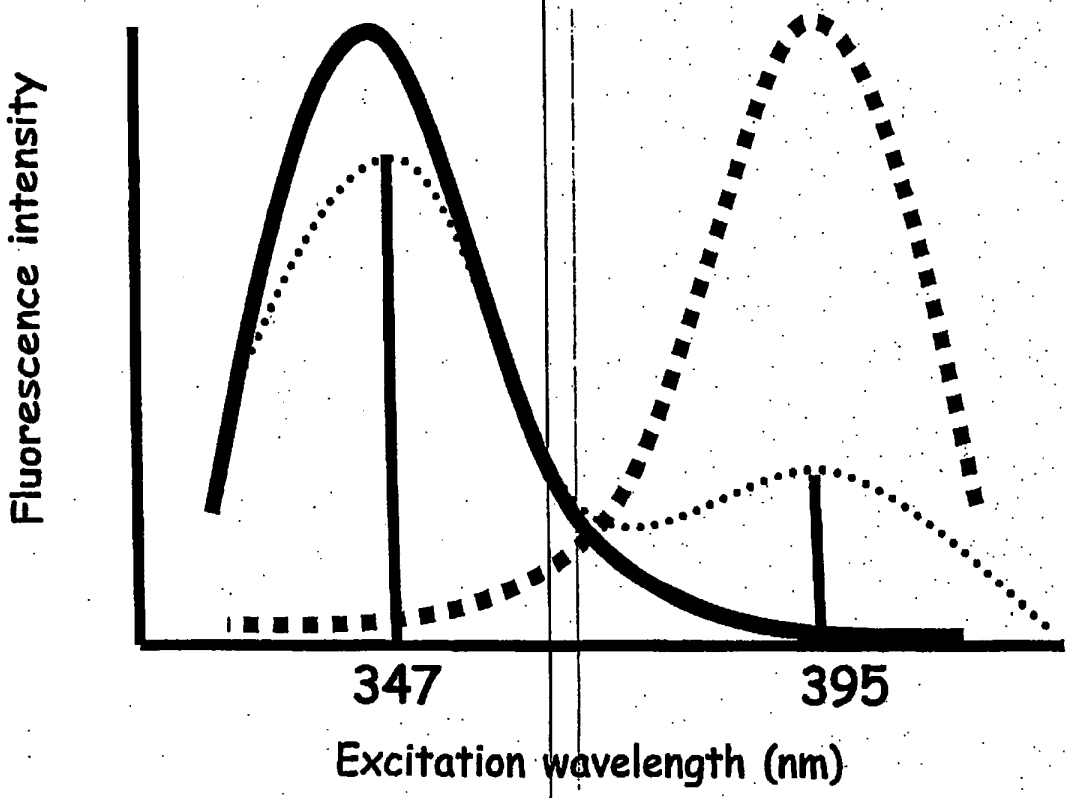
Abbreviations: AG-arabinogalactan; DEX-dextran; S-spermine.

Electrostatics of spermine conjugates determined trough covalently-attached hydroxycoumarin

pH<$pKa_{(HC)}$ protonated

pH>$pKa_{(HC)}$ unprotonated





Total probe = $I_{347}+I_{395}$

Dissociation degree (%) =

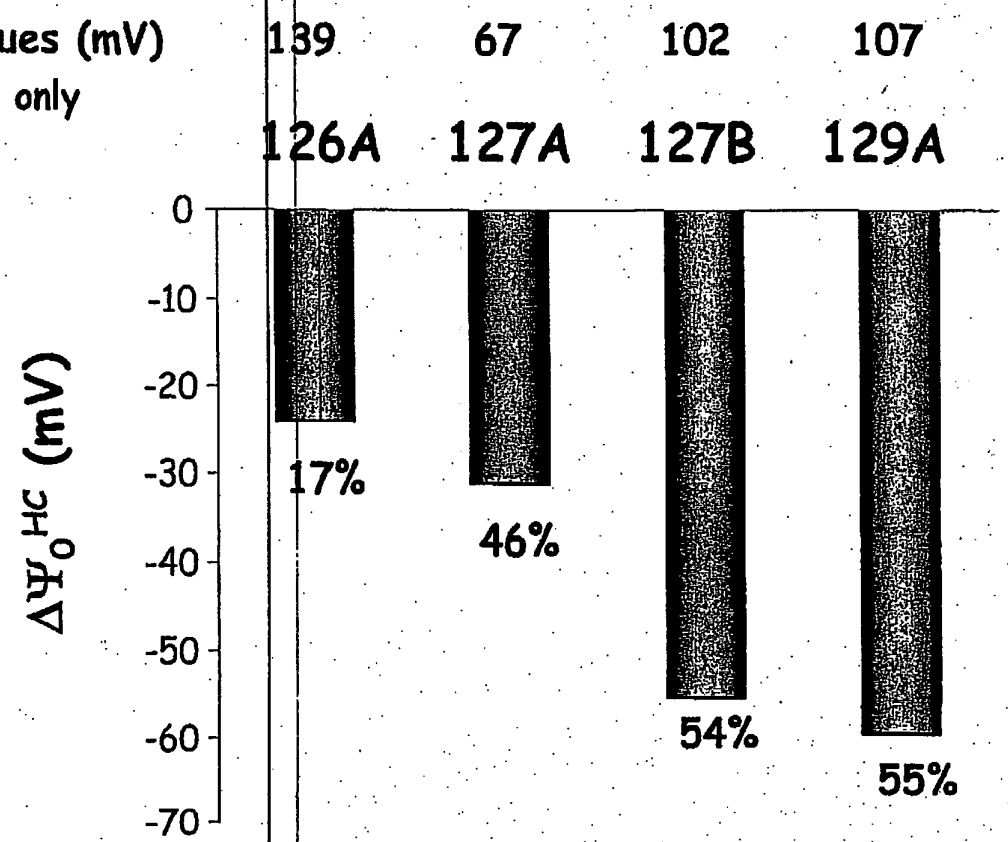
$I_{395}*100/(I_{347}+I_{395})$

<1% labeling

Electrostatics of polysaccharide spermine conjugates

polymer	Apparent pKa	Ψ_0 (mV)	$pH_{surface}$ 20 mM Hepes, pH 7.4
HC	7.8		
TA1-126A	5.4	139	9.8
TA1-127A	6.7	67	8.5
TA1-127B	6.0	102	9.2
TA1-129A	6.0	107	9.2
TA1-129B	6.7	64	8.5

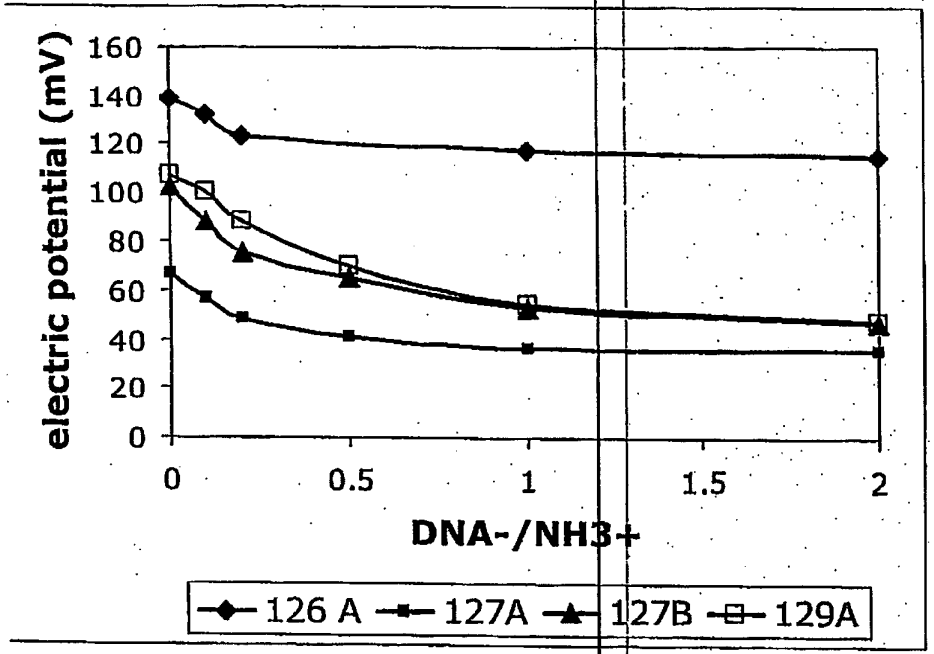
Electrostatic neutralization of polymers by DNA



Decreased electric surface potential upon DNA addition (charge ratio of 2.0 DNA^-/NH_3^+)

(B)

Electrostatic neutralization of polymers by DNA



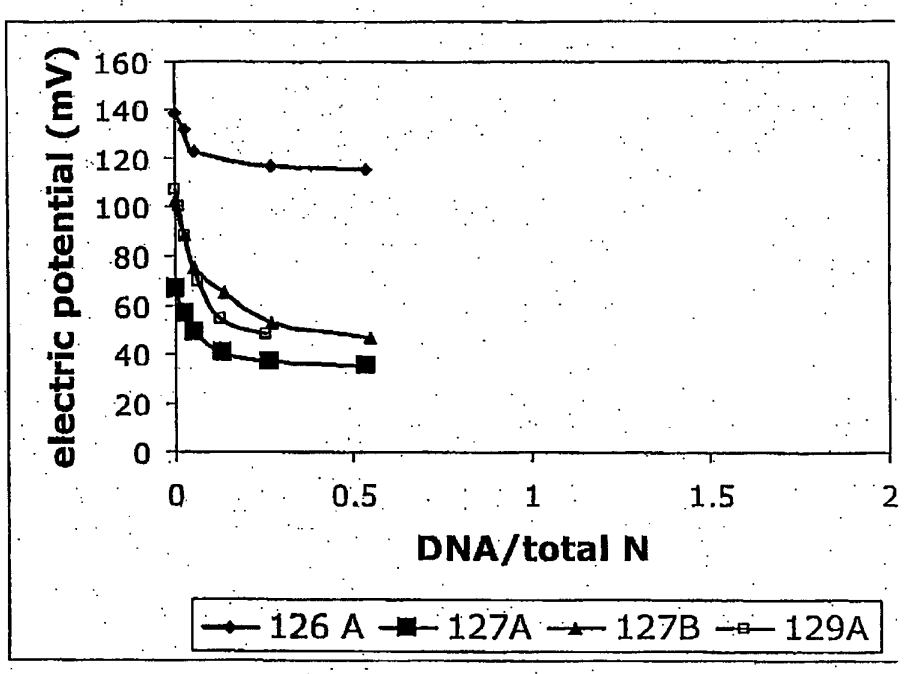


EXHIBIT C

DETECTION OF BINDING OF SPECIES TO PROTEIN-BASED SURFACES

FITC labeled albumin was obtained from Sigma (St Louis, Missouri, USA) (catalog number A 9771); the labeling was performed using FITC isomer I (Sigma catalog number F 7250). There are 9 moles FITC per mole albumin randomly attached to amino group of lysines of the albumin. FITC-albumin molecular weight is approximately 70000.

Fluorescence measurements were done using either a Perkin-Elmer LS 50B luminescence spectrometer (Norwalk, CT) or Synergy HT multi-detection microplate reader from Biotek (USA) at the excitation wavelength of 495 nm and emission wavelength of 520 nm (for Perkin Elmert) and filters of 485±20 nm and 528±20 nm for excitation and emission respectively (for Biotek).

1. pH Titration

The attached Figure shows that the fluorescence intensity is increasing with pH elevation in a sigmoid manner. pKa of 9.95 was assessed using Kaleidograph software version 3.6 indicating that on the average all FITC groups are in acidic (pH<7.0) environment.

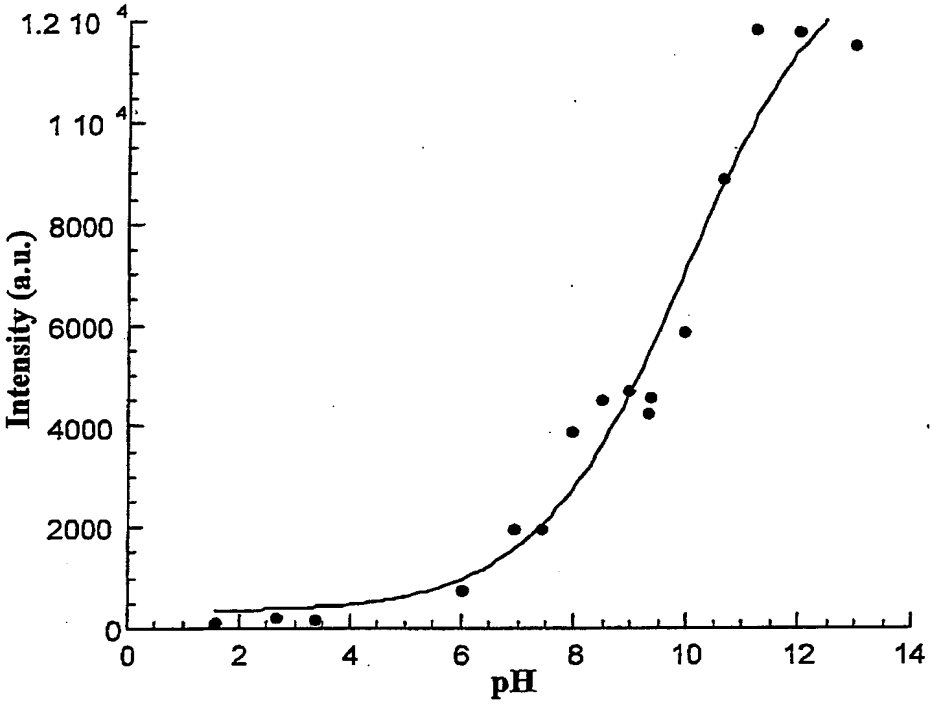
2. Effect of polymers and membranes assemblies on surface potential

When FITC-albumin was used as pH surface potential probe, large change (≤ 30%) in fluorescein fluorescence intensity occurred only when the cationic liposomes DOTAP or cationic polymers PEI or polylysine were used as test species. No change occurs when neutral or anionic liposomes were used.

It is well known that only when the pH sensitive fluorophore is in close proximity (<1.0 nm) with the macromolecule or the surface, the fluorophore will sense it [N. Zuidam and Y. Barenholz, Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin, Biochim. Biophys. Acta 1329 (1997) 211–222; N. Zuidam and Y. Barenholz, Electrostatic and structural properties of plasmid DNA–lipid complexes commonly used for gene delivery, Biochim. Biophys. Acta 1368 (1998) 115–128].

Thus, the conclusion drawn from the above results is that the binding of the cationic liposomes or cationic polymers (i.e. distance between the surface and test species of <1.0 nm) led to the change in fluorescence intensity.

Titration of Albumin-FITC



Handbook of Fluorescent Probes and Research Chemicals

Sixth Edition

by Richard P. Haugland, Ph.D.

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Table of Contents

Molecular Probes' *Handbook of Fluorescent Probes and Research Chemicals*

Chapter 1

Fluorophores and Their Amine-Reactive Derivatives

Molecular Probes

Contents

Related Chapters

1.1 Introduction to Amine Modification

Molecular Probes puts at your command a full spectrum of fluorophores and haptens for labeling biopolymers and derivatizing low molecular weight molecules. Chapters 1–5 describe the chemical and spectral properties of the reactive reagents we offer, whereas the remainder of this Handbook is primarily devoted to our diverse collection of fluorescent probes and their applications in cell biology, biochemistry, biophysics, microbiology, molecular biology, neuroscience and other areas.

Common Applications for Amine-Reactive Probes

Labeling Biopolymers

Amine-reactive probes are widely used to modify proteins, peptides, ligands, synthetic oligonucleotides and other biomolecules. In contrast to our thiol-reactive reagents (see Chapter 2), which frequently serve as probes of protein structure and function, amine-reactive dyes are most often used to prepare bioconjugates for immunochemistry, fluorescence *in situ* hybridization, cell tracing, receptor labeling and fluorescent analog cytochemistry.[1] In these applications, the stability of the chemical bond between the dye and biomolecule is particularly important because the conjugate is typically stored and used repeatedly over a relatively long period of time. Moreover, these conjugates are often subjected to rigorous hybridization and washing steps that demand a strong dye–biomolecule linkage.

Our selection of amine-reactive fluorophores for modifying biomolecules covers the entire visible spectrum (Table 1.1). An up-to-date bibliography is available upon request from our Technical Assistance Department or through our Web site (http://www.probes.com) for every amine-reactive probe for which we have references. Our Technical Assistance Department can also provide you with product-specific bibliographies, as well as keyword searches of the over 25,000 literature references in our extensive bibliography database. Included in Chapter 1 are discussions of the properties of Molecular Probes' most important proprietary fluorophores, including:

FluoReporter® Protein and Oligonucleotide Labeling Kits

Product (Catalog Number)	Features	Contents
FluoReporter Protein Labeling Kits • FITC (F-6434) • Fluorescein-EX (F-6433) • Oregon Green™ 488 (F-6153) • Oregon Green™ 500 (F-6154) • Oregon Green™ 514 (F-6155) • Rhodamine Red™-X (F-6161) • Tetramethylrhodamine (F-6163) • Texas Red®-X (F-6162)	The FluoReporter Protein Labeling Kits facilitate research-scale preparation of protein conjugates labeled with some of our best dyes. Typically, labeling and purifying conjugates with the FluoReporter Protein Labeling Kits can be completed in under three hours, with very little hands-on time. Each FluoReporter Protein Labeling Kit provides sufficient reagents for 5 to 10 labeling reactions of 0.2 to 2 mg of protein each.	• Five vials of the amine-reactive dye • Anhydrous DMSO • Reaction tubes, each containing a stir bar • Stop reagent • Ten spin columns • Collection tubes
FluoReporter Biotin-XX Protein Labeling Kit (F-2610)	This kit is designed for five biotinylation reactions, each with 5 to 20 mg of protein; up to 100 mg of protein may be labeled. A gel filtration column is provided for purifying the labeled proteins from excess biotin reagent. Once purified, the degree of biotinylation can be determined using the included avidin–biotin displacement assay; biotinylated goat IgG is provided as a control.	• Biotin-XX, succinimidyl ester • Anhydrous DMSO • A gel filtration column • Avidin–HABA complex • Biotinylated goat IgG
FluoReporter Mini-Biotin-XX Protein Labeling Kit (F-6347)	This kit permits efficient biotinylation of small amounts of antibodies or other proteins. The water-soluble biotin-XX sulfosuccinimidyl ester has a 14-atom spacer that enhances the binding of biotin derivatives to avidin's relatively deep binding sites. The ready-to-use spin columns provide a convenient method of purifying the biotinylated protein from excess reagents. Sufficient reagents are provided for five biotinylation reactions of 0.1 to 3 mg each.	• Biotin-XX, sulfosuccinimidyl ester • Reaction tubes, each containing a stir bar • Five spin columns • Collection tubes • Dialysis tubing
FluoReporter Biotin/DNP Protein Labeling Kit (F-6348)	The degree of biotinylation of proteins labeled with DNP-X–biocytin-X, succinimidyl ester can be assessed from the optical absorbance of DNP (ε = 15,000 $cm^{-1}M^{-1}$ at ~360 nm). The conjugates are recognized by both avidin derivatives and anti-DNP antibodies, permitting a choice of detection techniques. Sufficient reagents are supplied for 5 to 10 labeling reactions of 0.2 to 2 mg of protein each.	• DNP-X–biocytin-X, succinimidyl ester • Anhydrous DMSO • Reaction tubes • Stop reagent • Ten spin columns • Collection tubes
FluoReporter Oligonucleotide Amine Labeling Kits • Biotin-XX (F-6081) • BODIPY® FL (F-6079) • BODIPY® FL-X (F-6082) • BODIPY® R6G (F-6092) • BODIPY® TMR-X (F-6083) • BODIPY® 564/570 (F-6093) • BODIPY® 581/591 (F-6094) • BODIPY® TR-X (F-6084) • DNP-X (F-6085) • Fluorescein-X (F-6086) • Oregon Green™ 488 (F-6087) • Rhodamine Green™-X (F-6088) • Rhodamine Red™-X (F-6089) • Tetramethylrhodamine (F-6090) • Texas Red®-X (F-6091)	The FluoReporter Oligonucleotide Amine Labeling Kits permit the easy preparation of labeled oligonucleotides by reacting amine-derivatized oligonucleotides with a wide selection of our amine-reactive succinimidyl esters. The amine-reactive haptens and fluorophores in most of our 15 different FluoReporter Oligonucleotide Amine Labeling Kits contain aminohexanoic spacers ("X") to reduce the label's interaction with the oligonucleotide and enhance its accessibility to secondary detection reagents. The protocol has been optimized for labeling 5'-amino–modified oligonucleotides, 18 to 24 bases in length. Shorter or longer oligonucleotides may be labeled by the same procedure; however, adjustments to the protocol may be necessary. Sufficient reagents are provided in each kit for five complete labeling reactions of 100 µg of oligonucleotide each. The conjugates can be purified with our FluoReporter Labeled Oligonucleotide Purification Kit (F-6100; see below).	• Five vials of the amine-reactive label • Anhydrous DMSO • Labeling buffer • A detailed protocol for labeling
FluoReporter Oligonucleotide Phosphate Labeling Kits • Biotin-X-C_2 (F-6095) • BODIPY® FL-C_2 (F-6096) • BODIPY® TMR-C_2 (F-6097) • Rhodamine Red™-C_2 (F-6098) • Texas Red®-C_2 (F-6099)	These kits use proprietary coupling technology to link aliphatic amines to 5'-phosphate-terminated oligonucleotides to form phosphoramidate adducts. Unphosphorylated oligonucleotides can be enzymatically phosphorylated using T4 polynucleotide kinase prior to use of these labeling kits. Sufficient reagents are provided in each kit for five complete labeling reactions of 100 µg oligonucleotide each. The conjugates can be purified with our FluoReporter Labeled Oligonucleotide Purification Kit (F-6100; see below).	• Five vials of the phosphate-reactive label • Anhydrous DMSO • Labeling buffer • A detailed protocol for labeling
FluoReporter Labeled Oligonucleotide Purification Kit (F-6100)	The crude, labeled oligonucleotide is simply precipitated with ethanol to remove the excess reactive reagent, adsorbed on a spin column, washed to remove any unconjugated oligonucleotide and then eluted with an elution buffer to yield the conjugate. Isolated yields for the combined conjugation and purification steps are usually >50%, and the products are typically 90% pure as determined by HPLC. Conjugates can be used for most procedures without additional purification.	• Five spin columns • Buffers for column equilibration, loading, washing and elution • A detailed protocol